

Molecular Characterization of Imazaquin Tolerant and Sensitive Cowpea Genotypes

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Abstract: In this paper, to investigate the tolerance of some cowpea genotypes to imazaquin, seeds of 30 cowpea genotypes were treated with imazaquin at 0.06 kg active ingredient ha⁻¹ and control were treated with distilled water. Treated seeds were planted in plastic pots and raised for 3 weeks in a green house. Samples leaves were detached for DNA isolation at 2 weeks after sowing. Phenotypic result revealed that Hen-me and Hodi were the most tolerant (with 12.5% mortality rate) to imazaquin while Maptwapa and many others (with 100% mortality rate) were highly sensitive to imazaquin. However, there were no sequences differences between tolerant and sensitive genotypes in amplified region of the annotated portion of AHAS (acetohydroxy-acid synthase) from cowpea. It can be concluded that the major significant difference between imazaquin tolerance and sensitive cowpea is as a result of rapid metabolic detoxification of herbicides in tolerant cowpea. It is therefore imperative that enzymology involve in the differential metabolism of this herbicide in this crop needs urgent and necessary attention.

Keywords: AHAS Enzyme, Imazaquin, Herbicide Tolerance and Cowpea Genotypes

1. Introduction

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important grain legume crop grown in sub-Saharan Africa. Apart from its nutritional importance, it is also an important component in modern and traditional cropping systems [1]. It is widely use in crop rotation and intercropping with many cereals because of its nitrogen fixing ability. Almost all parts of the crop are useful. The seeds are used for human consumption while the haulm (stems and leaves) as animals feed [2]. The young pods and leaves are used as vegetables. Its ability to tolerate drought and do well under poor soil conditions, makes it a desirable crop in savanna ecology. Elsewhere cowpea is grown as cash a crop.

Imazaquin belongs to imidazolinones group of herbicides for broadleaf and grass weed control in soybean [3]. It can be applied as pre-plant incorporated, pre-emergence or post-emergence [4]. It is readily absorbed by root, foliage and rapidly translocated throughout the plant via xylem and phloem with accumulation in the meristematic tissue.

Imazaquin is well characterized in some species [like *Arbiodopsis*] [5] and attributed to specific base pair changes in acetohydroxy-acid synthase [AHAS] gene. Some cowpea genomic sequence is becoming available [6, 7] it will be of great interest to know if any mutations could be found between imazaquin tolerant and sensitive cowpea accessions.

Herbicide tolerance is the ability of the plant to grow and reproduce after herbicide application. Opinions differ on the basis of imazaquin tolerance in soybean. Tolerance of imazaquin to soybean may be due to rapid metabolism and degradation of the herbicide by the crop [8, 9]. Herbicide absorption and translocation may play minor role in imazaquin selectivity among some species [10]. Changes in coding sequence result in altering acetohydroxy-acid synthase (AHAS) enzymes which is the site of action of the herbicide may also result to imazaquin tolerance [11]. The phytotoxicity caused by imazaquin is due to inhibition of AHAS enzyme [3, 4, 9, 10, 11, 12]. AHAS is an enzyme that is involve in synthesis of branched-chain amino acid; isoleucine, leucine and valine. Imazaquin binds to the enzyme, causing dysfunction of the enzyme and reducing the

synthesis of isoleucine, leucine and valine that are necessary for protein synthesis. Plants resistant to acetolactate synthase (ALS) inhibiting herbicides have altered AHAS to which the herbicide does not bind [13]. Key amino acid residues within the coding site of the enzyme can protect it from imazaquin. AHAS from two lines of maize showed different spectra of herbicide tolerance compared with AHAS from sensitive maize [14]. Tolerance of cowpea to imazethapyr was due to an insensitive site of action, since extractable AHAS activity was reduced after imazethapyr application [10]. Herbicide tolerance is undesirable among weeds but desirable among crops. Herbicide tolerant crops offer a good opportunity for a farmer to control weeds. This is because of sufficient margin of selectivity between the crops and weeds such that the crop is not injured by the herbicides [15]. This gave way for new approach to herbicidal control of parasitic weed by treating the seeds of herbicide tolerant crops with a herbicide to control the obligate parasite. Use of herbicide seed treatments for parasitic weed control was first reported on cowpea in the year 1994 [16]. An ALS-inhibiting herbicidal seeds treatment was used to control *Striga hermonthica* in ALS-modified corn [16, 17, 18, 19]. Potentials of cinosulfuron and CGA152005 seed treatments for control of *Striga hermonthica* in upland rice was well documented [20]. In sorghum, seed treatments with acetolactate synthase (ALS) herbicide had successfully reduced *Striga* attachment to the host sorghum [15]. Herbicide seed treatments have become an important component of integrated weed management option. Identifying imazaquin tolerant cowpea can be an important land mark in the struggle to control *Striga* which is the most important biotic constraint to cowpea production in sub-Saharan Africa [21]. The goal was to identify imazaquin tolerant cowpea accessions and to attempt to characterize the molecular basis of such tolerance by comparing genomic sequence of annotated gene fragments of cowpea AHAS. Such information could be useful for developing imazaquin seed treatments for the control of *Striga* and other weeds in cowpea.

2. Material and Methods

2.1. Phenotyping of Cowpea for Imazaquin Tolerance

Table 1 provides the list of cowpea genotypes used in this study. They were obtained from US Department of Agriculture. Their seeds were imbibed with imazaquin at 0.06 kg active ingredient ha⁻¹ while the control was treated with distilled water. They were planted in a mixture of soils and compost and grown in the green house of the Department of Agronomy, Purdue University West Lafayette, Indiana USA. Data were taken on number of stands per pots at 3 weeks after sowing. Mortality rate of herbicide treated genotypes were calculated as (Number of survived stand per pot at 3 weeks/ Number of planted seeds per pot) X 100. Genotypes that recorded 70% ($\geq 30\%$ mortality rate) and above survival rates are considered as tolerant while less than 50% ($\geq 50\%$) are considered as sensitive. Young leaves from

two week old plants were harvested and stored at -80°C until used for DNA isolation.

Table 1. Cowpea Genotypes Used in the Molecular Study.

Genotypes	Country of origin	100-seeds weight(g)
Dan arba'in	Nigeria	118.12
Hen-me	Srilanka	62.25
Hodi	Srilanka	57.50
H.v.s 6/6	Uganda	71.12
Kibarani	Kenya	54.12
Matwapa-1	Kenya	92.12
Matwapa-2	Kenya	55.00
Sokoto N	Nigeria	108.12
Tvu 1392	Kenya	70.25
Tvu 1457	Kenya	80.37
Tvu 1542	Nigeria	74.25
Tvu 1638	Kenya	65.12
Tvu 1647	Tanzania	73.50
Tvu 1771	Nigeria	93.75
Tvu 1773	Brazil, Rondonia	82.75
Tvu 1801	Kenya	66.12
Tvu 1802	Tanzania	71.62
Tvu 1803	Uganda	77.37
Tvu 1908	Nigeria	57.37
Tvu 1977	Zambia	67.87
Tvu 1998	Kenya	54.37
Tvu 2003	Tanzania	78.62
Tvu 2004	Tanzania	71.37
Tvu 2018	Tanzania	72.00
UCR 236	Tanzania	67.12
UCR 237	Tanzania	67.00
UCR 242	Tanzania	73.25
UCR 243	Tanzania	104.00
V.u.6	Kenya	71.00
t318	Kenya	63.75

2.2. Genomic DNA Isolation

Total DNA was isolated from the leaf tissues using DNA extraction kit (QIAGEN, USA) according to the manufacturer's protocol. The isolated DNA was dried at room temperature for 1 hour, dissolved in 100 μ l and quantified with spectrophotometer ND-100 (Thermo scientific, wilmigton DE, USA) and stored at 4°C.

2.3. Primer Selection and Preparation

The PCR primers used in the study were derived from cowpea Gene Space Read (GSR) sequences annotated for AHAS enzyme inhibitor from the Cowpea Genomic Knowledge Base (CGKB) website; (<http://cowpeagenomics.med.virginia.edu/CGKB>). CGKB is an annotation knowledge base developed under Cowpea Genomics Initiative (CGI). The database is based on information derived from 298,848 cowpea gene space sequences (GSS) isolated by methylation filtering of genomic DNA [5]. It consists of three knowledge bases: GSS annotation and comparative genomic knowledge base, GSS enzyme and metabolic pathway knowledge base, and simple sequences repeats (SSRs) knowledge base for molecular discovery.

The primers were synthesized by IDT Integrated Technologies, USA. The name of primers and their nucleotide sequences and sizes are presented in Table 2. The

primers were screened for their ability to detect polymorphisms between tolerance and sensitive genotypes. Extracted DNA samples of Hen-me and Hodi were used as imazaquin tolerant while TVU 1778 and TVU 1802 were considered as sensitive genotypes.

2.4. PCR

Both forward and reverse primers were diluted with water base on their weight. 10 µl of each primer solution was pipetted and diluted with 90 µl of distilled water. 1.5 µl each of forward and reverse primers was pipetted and mixed with 1.0 µl of extracted DNA solution and 21 µl of MangoMix (Bioline) in 0.2 ml PCR tube to make reaction volume of 25 µl.

The PCR tubes were loaded on to thermo cycler and runs for 35 circles of 30 seconds at 94°C, 35 seconds at 55° and 1.30mn at 72°C for complete 2 hours. 10 µl of amplified PCR product were resolved on 5% agarose gel using 0.5 x TBE containing Gel red with horizontal electrophoresis apparatus at 60 V for 2 hours. The gel was photographed using UV transillumination. The size of DNA bands in base pairs was determined using 1 kb ladder as standard marker. Four primers pairs were used to amplify DNA samples of sensitive and tolerant cowpea genotypes.

2.5. Genes Sequencing

PCR products were purified using standard procedure prescribed by QIAquick PCR purification kit according to manufacturer's recommendation. The purified DNA

undergone sequencing procedures performed at Purdue Genomics core facility. Sequences were then analyzed and aligned with Codon Code Aligner using MEGA3 software.

3. Results

3.1. Phenotyping 30 Diverse Cowpea Genotypes for Imazaquin Tolerance

Based on percentage plant mortality, there were some differences in genotypic response to imazaquin tolerance at 3 weeks after sowing (Figure 1). Genotypes Hen-me and Hodi were the most tolerant with 12.5% mortality rate each. Matwapa 1 & 2, TVU 1392, TVU 1773 and many others were among the most sensitive genotypes with 100% mortality rate. Other genotypes exhibit low to intermediate level of tolerance.

3.2. Product Form

Figure 2 showed the size of band produce by each primer size. Five primers (62.5%) produce single band within the predicted size range of 400-500 bp (figure 2).

3.3. Sequencing

Figure 3 showed the amplified region of the cowpea AHAS annotated by CGKB. No polymorphisms were seen in this region between imazaquin tolerant and sensitive accessions.

Table 2. List of PCR Primers used Showing the Nucleotide Sequence and the Sizes.

Primer	Primer sequence	Product size (bp) Amplicon
VuAHAS_1-F	5'- TCG GGT AGC AGA GGA GTT TT -3'	575
VuAHAS_1-R	5'- TTG CAG ATG CTT GTG GGA TA -3'	
VuAHAS_2-F	5'- GAT TGA ATC CTG CTT CCT TCA -3'	548
VuAHAS_2-R	5'- GCC AAC AGA GCT CAC ACG TA -3'	
VuAHAS_4-F	5'- TTC CTC AAT AAG CAA CCA TGA A -3'	564
VuAHAS_4-R	5'- CAG TGG GAG GAT CGG TTC TA -3'	
VuAHAS_5-F	5'- TGC ATT GGT ACG AAG AAA TCT G -3'	507
VuAHAS_5-R	5'- GGG ATA CCA TCA GCT CGT GT -3'	

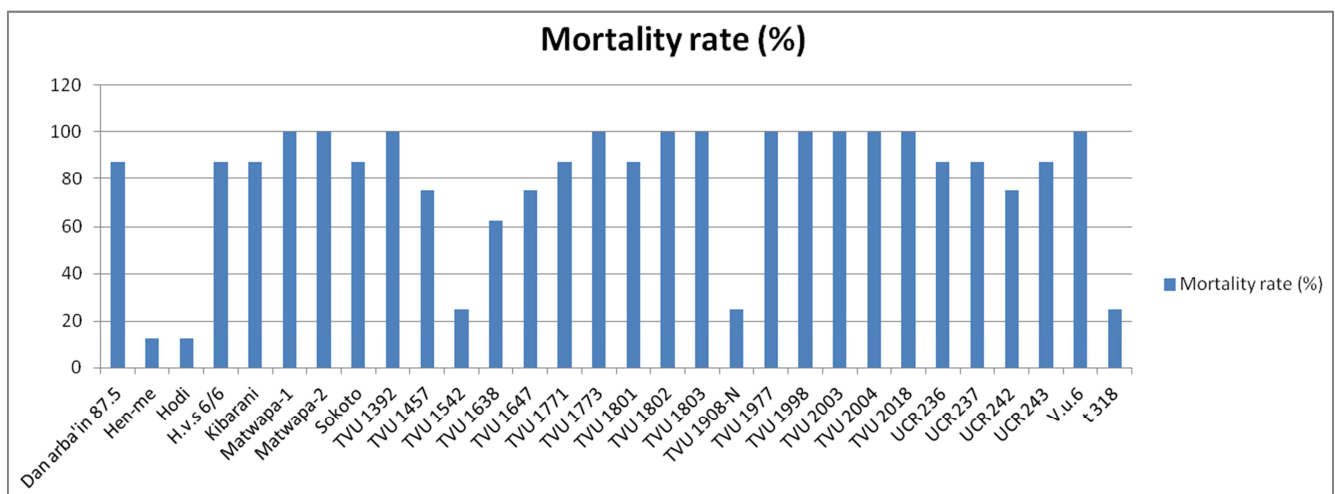


Figure 1. Mortality rate of cowpea genotypes treated with imazaquin.

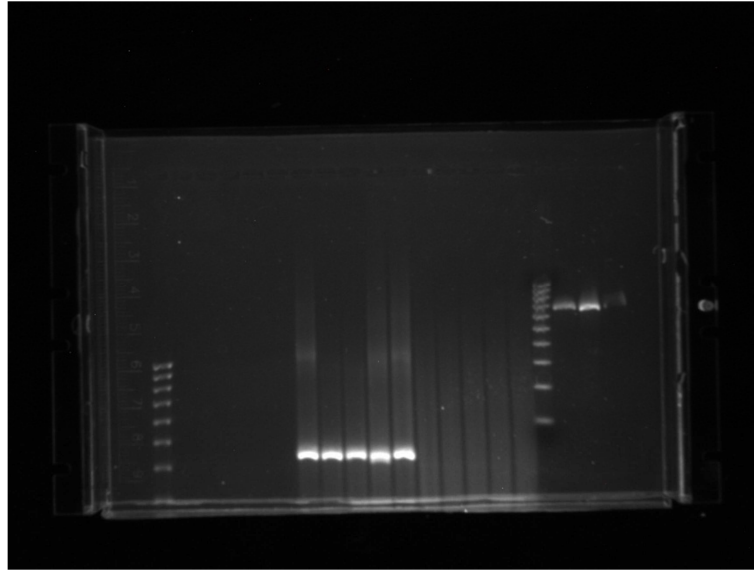


Figure 2. Agarose gel electrophoretic analysis of PCR amplified primers using the 4 primer pairs combination and DNA from imazaquin tolerant and sensitive genotypes.

T_50*	GAATGTGCATGTTTTCAITTCATTTTGATTGAATCCTGCTTCCTTCAATAGATTCTGCTT	60
C_83**	GAATGTGCATGTTTTCAITTCATTTTGATTGAATCCTGCTTCCTTCAATAGATTCTGCTT	60
Gn1_939284***	GAATGTGCATGTTTTCAITTCATTTTGATTGAATCCTGCTTCCTTCAATAGATTCTGCTT	60

T_50	ATACTTGTGACAGAAATAGTATGCTTGCATATCATTTTCAGTCTGATACTACAGAAAAA	120
C_83	ATACTTGTGACAGAAATAGTATGCTTGCATATCATTTTCAGTCTGATACTACAGAAAAA	120
Gn1_939284	ATACTTGTGACAGAAATAGTATGCTTGCATATCATTTTCAGTCTGATACTACAGAAAAA	120

T_50	CCAAAGGCTGCAGTACATAAAACAGGGGATAGACGCCACTGCATATGACAAGTTAAATAA	180
C_83	CCAAAGGCTGCAGTACATAAAACAGGGGATAGACGCCACTGCATATGACAAGTTAAATAA	180
Gn1_939284	CCAAAGGCTGCAGTACATAAAACAGGGGATAGACGCCACTGCATATGACAAGTTAAATAA	180

T_50	AAGGGGAAATACATAATCTTGCTACCAAACTACTACAGAGGCCAAGCCTGGAACTAG	240
C_83	AAGGGGAAATACATAATCTTGCTACCAAACTACTACAGAGGCCAAGCCTGGAACTAG	240
Gn1_939284	AAGGGGAAATACATAATCTTGCTACCAAACTACTACAGAGGCCAAGCCTGGAACTAG	240

T_50	AAAAGCACTATCTTTTATACATTGAACAAAACAAGCATGAGGGAATATTGGTGTTGACA	300
C_83	AAAAGCACTATCTTTTATACATTGAACAAAACAAGCATGAGGGAATATTGGTGTTGACA	300
Gn1_939284	AAAAGCACTATCTTTTATACATTGAACAAAACAAGCATGAGGGAATATTGGTGTTGACA	300

T_50	ATCAGTACCTAGTTCTACCATCACCCTCAGTAATGACATCATTGAAGGATCCATTACTGG	360
C_83	ATCAGTACCTAGTTCTACCATCACCCTCAGTAATGACATCATTGAAGGATCCATTACTGG	360
Gn1_939284	ATCAGTACCTAGTTCTACCATCACCCTCAGTAATGACATCATTGAAGGATCCATTACTGG	360

T_50	GAATCATGGGCAGCACGTGTTCTGTGATGAGGCACTATGACCTCAAGAGATAGGGGCCAG	420
C_83	GAATCATGGGCAGCACGTGTTCTGTGATGAGGCACTATGACCTCAAGAGATAGGGGCCAG	420
Gn1_939284	GAATCATGGGCAGCACGTGTTCTGTGATGAGGCACTATGACCTCAAGAGATAGGGGCCAG	420

T_50	GGGTGTCCAGCATTTTCTGAATTGCCATCTAAGCTCTTCTTCTTCGTACACGAACCC	480
C_83	GGGTGTCCAGCATTTTCTGAATTGCCATCTAAGCTCTTCTTCTTCGTACACGAACCC	480
Gn1_939284	GGGTGTCCAGCATTTTCTGAATTGCCATCTAAGCTCTTCTTCTTCGTACACGAACCC	480

T_50	CGGGTATCCCAAGCATCTGCAAACTTTAGCAATGTTTGGGAATATCT-----	529
C_83	CGGGTATCCCAAGCATCTGCAAACTTTAGCAATGTTTGGGAATATCT-----	529
Gn1_939284	CGGGTATCCCAAGCATCTGCAAACTTTAGCAATGTTTGGGAATATCTCATCTCCCTC	539

T_50	-----	
C_83	-----	
Gn1_939284	GAGGGATCTCCAGATACGTGTGAGCTCTGTTGGCTTGTAGAACCGATCCTCCACTGA	599

T_50	-----	
C_83	-----	
Gn1_939284	ACCACCATACCAAGTGCTGATTATCAACAACATATCTTAACAGGGAGAT	651

Figure 3. Sequencing of imazaquin tolerant and sensitive cowpea genotypes base on amplified region of the annotated AHAS portion from cowpea.*= Imazaquin tolerant genotype; **= Imazaquin sensitive genotype; ***= Annotated AHAS gene.

4. Discussion

Absence of polymorphism between imazaquin tolerant and sensitive cowpea genotypes suggested that tolerance may not be due to inhibition of AHAS enzyme. It was earlier reported that imazaquin tolerance in soybean was due to changes in coding sequence resulting in altering AHAS (acetohydroxy-acid synthase) enzymes which is the site of action of the herbicide [5]. It was also noted that differential target site sensitivity is not the bases of soybean tolerance to imazaquin since AHAS from soybean and other sensitive plant are equally sensitive to herbicide [14]. The absent of sequences in amplified region of the annotated portion of AHAS enzyme from cowpea could also be attributed to fact that the genes may belong to the same family or they are the same genes but deposited with different name. It may be possible that there may be difference elsewhere in the gene outside the amplified region. For example, from bp 518 to bp 651 were not amplified by the primers, it is possible that there could a nucleotide polymorphism within these unamplified regions of the DNA.

Tolerance of Hen-me, Hodi, TVU 1542, TVU 1908 and t318 to imazaquin (base on phenotype) could be attributed to gradual metabolism of the herbicide. It has been reported that plants differ in their ability to compensate for the damaging effects of the herbicide with no physiological mechanism involved [22]. The differences in tolerance to imazaquin among plants species are caused by variations in the rate of metabolism of the parent herbicide [3]. Some soybean cultivars have displayed differential tolerance to imazaquin [23]. Selectivity of imazaquin to soybean was reported to be due to rapid metabolism and degradation of the herbicide by soybean plant [9]. Susceptible plants are either unable to metabolized the herbicide or rate of metabolism is very low. Crop tolerance to herbicides can also result from such factors as reduced absorption and translocation. Differential target site sensitivity may therefore not be the only basis for cowpea tolerant to imazaquin since there is no sequences difference in amplified region of the annotated portion of AHAS cowpea. It is possible that changes in coding sequence of AHAS enzymes play a significant role in herbicide resistance (which is inherited ability of plant to survive and reproduce following exposure to a dose of herbicide that would normally be lethal to the wild type) but not tolerance.

5. Conclusion

It can be concluded that the major significant difference between imazaquin tolerance and sensitive cowpea is as a result of rapid metabolic detoxification of herbicides in tolerant cowpea. It is therefore imperative that enzymology involve in the differential metabolism of this herbicide in this crop needs urgent and necessary attention.

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